

Supplemental Material:

Detailed Methods

Mice

All animal protocols were approved by the Animal Care and Use Committee at the University of Virginia. *Id3^{fl/fl}* mice were a generous gift of Dr. Yuan Zhang (Duke University). *CD19^{Cre/+}* mice and *Rag1^{-/-}* mice were provided by Timothy Bender (University of Virginia). *Apoe^{-/-}* mice were purchased from Jackson Laboratory. *Id3^{fl/fl}* mice were bred to the *Apoe^{-/-}* line and then with *CD19^{Cre/+}* mice to develop B cell specific *Id3* knockouts as previously described ¹. *Rag1^{-/-}* mice were bred with *Apoe^{-/-}* to generate *Rag1^{-/-}Apoe^{-/-}* mice. All mice, purchased or generated, were backcrossed at least 10 generations to C57BL/6J mice. Mice were fed either a standard chow diet or Western Diet (Tekland, 7012 or TD.88137). Mice were euthanized in all experiments by CO₂ asphyxiation. Only male mice were used for all experiments.

Serum Cholesterol Determination

Cholesterol levels were determined as previously described by the University of Virginia Medical laboratories ².

Analysis of atherosclerotic lesions

Hearts were removed and prepared as previously described ³. Briefly, mice were perfused by left ventricular puncture with heparinated PBS to avoid clotting. Hearts were separated from the aorta distal to the aortic sinus. The lower third of the heart was removed by scalpel and the remaining heart was vertically embedded with cut edge down in OCT compound (Tissue-Tek) then wrapped in aluminum foil and snap frozen by floating the mold on top of liquid nitrogen for 60 seconds. Blocks were then left on dry ice to completely freeze and stored at -80°C. Serial 5µm sections were cut by Cryostat (Leica biosystems) from the beginning of the three aortic leaflets to the aortic arch. Slides were stored at -80°C until stained. For lesion analysis, 1/4-1/5 of the total slides, equally spaced, were stained with Oil Red O lipid stain and counterstained with hemotoxylin (both from Sigma). Sections were imaged using an Olympus BX51 high magnification light microscope. Aortas were prepared as previously described ². Briefly, aortas were fixed in 4% paraformaldehyde then opened longitudinally, pinned, and stained using Sudan IV (Sigma). Aortas were imaged with a Nikon D70 DSLR camera. Plaque areas were assessed using Image-Pro Plus software (Media Cybernetics). For aortic sinus measurements, maximum plaque area measured from each mouse was used for comparison. For aortic plaque measurements, total percentage of the aorta that was covered by plaque was used for comparison.

Immunofluorescence and Tunel analysis of aortic sinus sections

Slides of the aortic sinus, described above, were stained for macrophage content as previously described ² using biotinylated Mac-2 as the primary antibody (Cedarlane CL8942B) and Streptavidin Alexa Fluor 488 as the secondary antibody (Invitrogen Molecular Probes S11223) then counterstained with DAPI and mounted (Vectashield H-1500). For staining of apoptotic cell bodies the TUNEL method was used following the protocol from ApopTag Peroxidase In Situ Apoptosis Detection Kit (emdMillipore S7100). Imaging for both was done using an Olympus BX51 high magnification light microscope. Images were analyzed using ImageJ (<http://imagej.nih.gov/ij/>).

Adoptive transfer of B-1b cells into Rag1^{-/-}Apoe^{-/-} hosts

Following electronic cell sorting, as described below, 1×10^5 B-1b cells were transferred interperitoneally (IP) into 8-week-old *Rag1^{-/-}Apoe^{-/-}* mice. Mice were maintained on chow diet for one week following transfer then switched to Western Diet for 16 weeks at the end of which time the animals were euthanized and hearts were collected for histological analysis of atherosclerotic lesions within the aortic roots as described above.

Immunizations

8-10-week-old male *Id3^{BKO}Apoe^{-/-}* and *Id3^{WT}Apoe^{-/-}* mice were immunized with the T cell dependent antigen DNP-KLH as described previously⁴. Briefly, mice were immunized IP with 100 μ g DNP-KLH in complete Freund's adjuvant or adjuvant alone as control then boosted with 100 μ g DNP-KLH on day 21 in PBS or PBS alone as control. Blood was collected on days 0 (prior to immunization), 7, and 21 (prior to boost). On day 28 mice were euthanized and blood was collected by cardiac puncture. Bone marrow was harvested and treated, as described below, for flow cytometric analysis of plasma cells (PC).

Preparation of tissues for Flow Cytometry and Cell Sorting

PerC cells, splenocytes, PBMCs and bone marrow (BM) cells were harvested and single cell suspension were prepared as previously described^{2,5}. Cells were blocked for Fc receptors using anti-FC γ RIII/II (CD16/32, FCR-4G8, Life Tech) then stained for cell surface markers using fluorescently-conjugated antibodies for 20 minutes at 4°C. Cells were washed in PBS and stained with a fixable live/dead stain diluted in PBS for 20 minutes at 20°C then fixed in 2% PFA in PBS for 10 minutes prior to resuspending in FACS buffer (PBS with 0.05% NAN₃ and 1% BSA). For FAC sorting, cells were resuspended in modified FACS buffer (PBS with 1% BSA) and 4',6-Diamidino-2-Phenylindole (DAPI) live/dead stain then immediately taken to sorting facility. B-1b cells were sorted to better than 99% purity from their parent gate. Flow cytometry antibodies: CD5 (PE, 53-7.3), CD19 (PECy7 or APCef780, 1D3), CD21 (APC, 8D9, 7G6), CD23 (PECy7, B3B4), CD43 (FITC, S7), B220/CD45R (APC, RA3-6B2), IgD (FITC, 11-26.2a), and IgM (e450 or FITC, II/41, R6-60.2), Ter119 (Biotin, Ter119), NK1.1 (Biotin, PK136), Gr-1 (Biotin, RB6-8C5), CD11b (Biotin, M1/70), CD138 (PE, 281-2). All antibodies were purchased from eBioscience, BD Bioscience, and Biolegend. Immunoglobulin isotype antibodies used for plasma cell intracellular staining were conjugated to FITC and purchased from Southern Biotech (IgG1 clone SB77e, IgG2a clone SB84a, IgA clone 11-44-2), BD (IgG2c clone R19-15) and Abcam (IgG3 clone ab97259). Intracellular staining for immunoglobulin isotypes was done using reagents and protocols from the BD Cytofix/Cytoperm kit (BD, 554714). Live/Dead discrimination was determined by LIVE/DEAD fixable yellow staining (Invitrogen) or DAPI. For flow cytometry of human samples, PBMCs were isolated from blood using SepMate tubes per the manufacturer's instructions (Stemcell Technologies, 15425). Briefly, blood was diluted with an equal volume of wash buffer: PBS with 2% FBS (Gibco). The diluted sample was carefully added down the side of the SepMate tube containing Ficoll-Paque Plus (GE Healthcare, 17-1440-02). Tubes were centrifuged at 1200g for 10 minutes then the buffy coat layer was removed, washed and resuspended for staining. Antibodies were from BD – CD3 (PE-CF594, SK7), CD27 (BV421, M-T271), and eBioscience – CD20 (APC-H7, 2H7), CD43 (FITC, 84-3C1). Electronic cell sorting was carried out at the University of Virginia Flow Cytometry Core on an Influx cell sorter (BD Bioscience) using BD FACS Software Sorter Software. Immunophenotyping was performed on a CyAn ADP (Beckman Coulter) and analyzed with FlowJo software (Tree Star Inc). All gates were determined using fluorescence minus one (FMO) controls.

In vitro stimulation assays

Post electronic cell sorting, *Apoe*^{-/-} B-1b cells were plated at $1-4 \times 10^4$ cells per well in a 96 well plate in 200 μ l of B cell culture media: complete RPMI 1640 containing 10% heat inactivated FBS (Hyclone), 10mM HEPES, 1x nonessential amino acids, 1mM sodium pyruvate, 100 U/mL penicillin and 100 mg/mL streptomycin, 0.55 mM 2-Mercaptoethanol with 50 μ g/mL LPS (Sigma, L4391), 100nM CPG (ODN 1668, Invivogen), or PBS for 72 hours. All culture reagents are from Gibco unless otherwise specified. Media and cells were taken up and centrifuged. The supernatant was collected for measurement of immunoglobulins by ELISA.

Enzyme-linked Immunosorbent Assay (ELISA)

Specific Ab levels to given antigens in plasma from mice were determined by chemiluminescent ELISA as previously described^{6,7}. In brief, Microfluor® 2 White "U" Bottom Microtiter® plates (Thermo Labsystems, Franklin, MA, USA) were coated with various antigens at 5 μ g/mL PBS overnight at 4°C. The plates were blocked with 1% BSA in TBS, serially diluted plasma was added, and the plates incubated for 1.5h at room temperature. Bound plasma Ig isotype levels were detected with various anti-mouse Ig isotype-specific alkaline phosphatase (AP) conjugates using LumiPhos 530 solution, and a Dynex Luminometer (Dynex Technologies, Chantilly, VA, USA). The following goat AP-conjugated secondary Ig isotype-specific Abs were used; anti-mouse IgM (μ -chain specific) (Sigma-Aldrich) and anti-mouse IgG1, IgG2c, and IgG3 (all Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA). Data were expressed as relative light units counted per 100 milliseconds (RLU/100 ms) and converted to absolute Ig values from simultaneously run standard curves for each given isotype. For each set of mice, plasma pools were made and used for formal dilution curves to determine optimal dilution for each antigen to use in binding assays. A specific non-saturating dilution was chosen for each antigen, and then plasma samples from each mouse were assayed to determine mean and SD for that determination. Antigens used were prepared as previously described. IgM E06 titers were determined by plating AB1-2, a T15-antiidiotypic, and then determining the amount of IgM bound to AB1-2 using the anti-mouse IgM as above.

Ab levels against MDA-LDL and apoB-immune complexes (ICs) in human plasma were measured by chemiluminescent ELISA as previously described⁸. Briefly, MDA-LDL (5 μ g/ml) was coated on microtiter well plates, plasma was added, and IgG or IgM antibodies binding to MDA-LDL was detected with alkaline phosphatase labeled goat anti-human IgG or (Sigma). ApoB-100 ICs were detected by plating murine monoclonal antibody MB47 to bind a saturating amount of human apoB. Plasma was added and IgG or IgM antibodies binding to the captured apoB were detected with alkaline phosphatase labeled goat anti-human IgG or IgM.

Antibody titers against DNP were measured as previously described⁴. Briefly, 96 well plates were coated with DNP-BSA (10 μ g/ml) overnight. Samples were incubated on plates for 2 hours then washed and alkaline phosphatase conjugated, isotype specific, secondary antibodies were added for 2 hours then DnPP reagent was added. The mean optical density at 450 nm was measured and compared to a standard curve developed from pooled serum in order to determine RLU/100ms

ELISPOT

Single cell suspension of BM was prepared as described above. Sterile MultiScreen IP-Plates (Millipore, MSIPS4510) were used for the assay according to manufacturer's protocol. Wells were coated with unlabelled anti-mouse IgM antibody (Southern Biotech) and incubated overnight at 4°C. The following day the antibody solution was removed and the membrane was washed and then blocked with RPMI 1640+10% FCS for 2 hours at 37°C. A suspension of 1×10^6 cells/ml was prepared then 2.5×10^5 cells were added to the first well then serially diluted

for each subsequent well incubated overnight at 37°C in a cell culture incubator (5% CO₂). Cells were decanted then biotin-labeled anti-mouse IgM antibody (1:5000 dilution) was added to each well and incubated 2 hours. Following washing streptavidin alkaline phosphatase (Abcam) was added and incubated 30 min at room temp. Again following washing BCIP/NBT was added and incubated until spots became visible. Each spot on the membrane indicated an antibody secreting cell. Wells were imaged under a dissecting microscope (Zeiss) then spots were counted manually. The ideal concentration of cell was determined based on visible spots for counting.

Human Genotyping

Id3 SNP (rs11574, Assay ID# C_2462609_10) genotyping was performed using the ABI Taqman SNP Genotyping assay from LifeTechnologies. Briefly, DNA was isolated from whole blood using the Gentra Puregene kit (Qiagen) according to the manufacturer's instructions. PCR was performed on the ABI 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA) in 5 µL reaction volume. For each PCR, 1 µL genomic DNA (~10 ng) was mixed with 2.5 µL 2× TaqMan Genotyping Master Mix (P/N 4371355), 0.25 µL 20× TaqMan SNP genotyping assay mix and 1.25 µL of nuclease free water. Assays were loaded onto 384 well plates (Life Technologies, 4309849) and PCR conditions were one cycle at 95 °C for 10 min, 40 cycles at 92 °C for 15 s, 60 °C for 1 min. All genotypes were analyzed and assigned automatically using the ABI SDS 2.3 software.

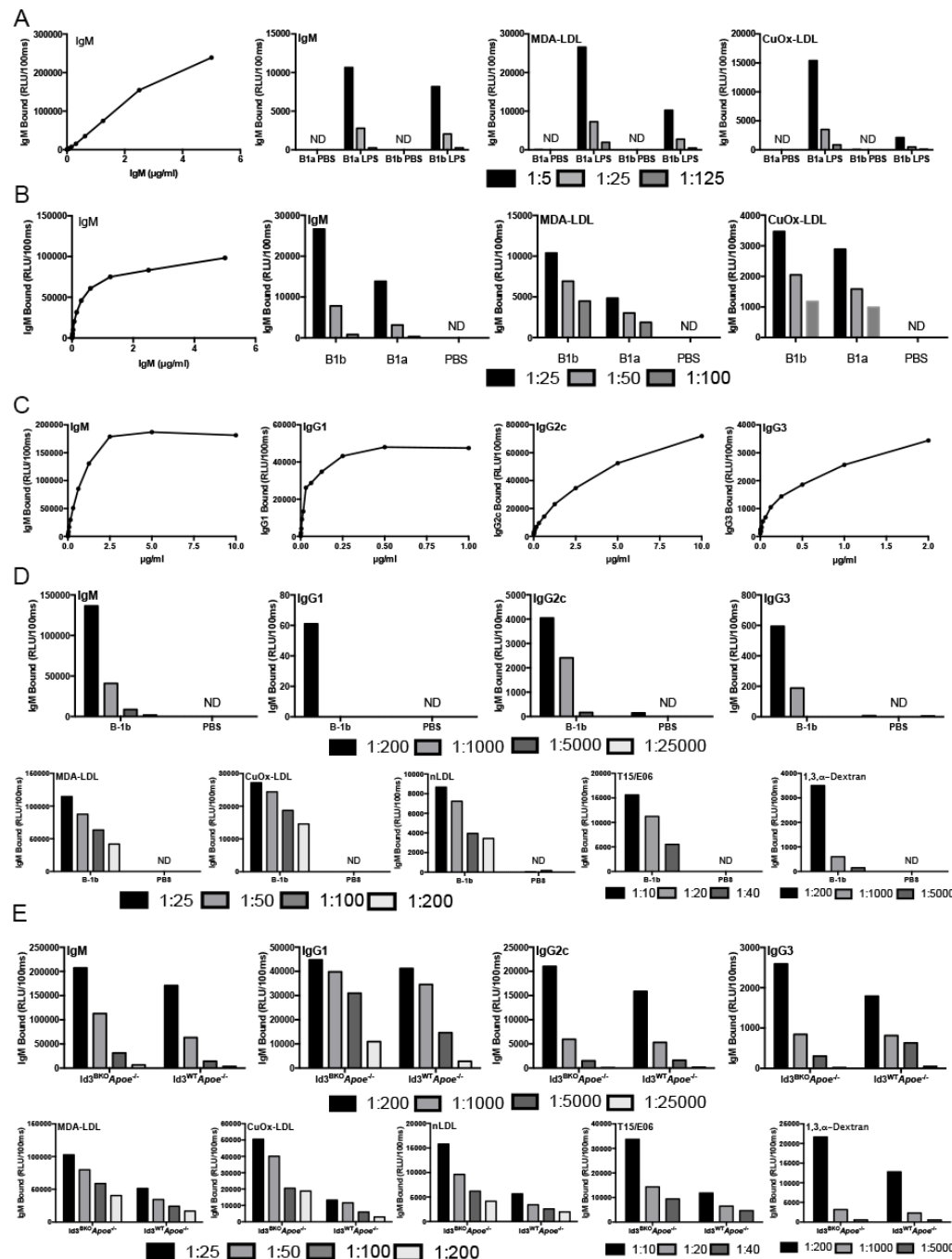
Real time PCR

Total RNA was isolated from FACS purified peritoneal B cells using the RNeasy Plus Micro kit with gDNA elimination columns (Qiagen). RNA (1 µg) was reversed transcribed with SuperScript III First-Strand Synthesis System by Oligo d(T) tailing (Invitrogen). Secreted IgM was normalized by the $\Delta\Delta C_q$ method to 18S with SYBR® Green real-time PCR (SsoFast™ EvaGreen® Supermix, Bio-Rad). PCR reactions were always performed with at least duplicate wells using the C1000 Thermal Cycler and CFX96 Real Time system (Bio-Rad). Primers were used as follows: slgM, forward primer (5'-GGA GAG ACC TAT ACC TGT GTT GTA GG-3') and reverse primer (5'-TGA GCG CTA GCA TGG TCA ATA GCA G-3'); 18S forward primer (5'-CGG CTA CCA CAT CCA AGG AA-3'), reverse primer (5'-AGC TGG AAT TAC CGC GGC-3').

Statistical Methods

To test if data sets fit a Gaussian distribution, a D'Agostino-Pearson omnibus normality test was used. If data was normal, a two-tailed student's t-test was performed. For non-Gaussian distributed data, a Mann-Whitney test was performed to determine statistical significance. To compare differences in more than two data sets, a one-way analysis of variance (ANOVA) and Holm-Sidak or Tukey's multiple comparisons test were used. Data was analyzed using Prism 6.0b (GraphPad Software, Inc.). Results are displayed containing all replicated experiments and values shown are mean ± SEM.

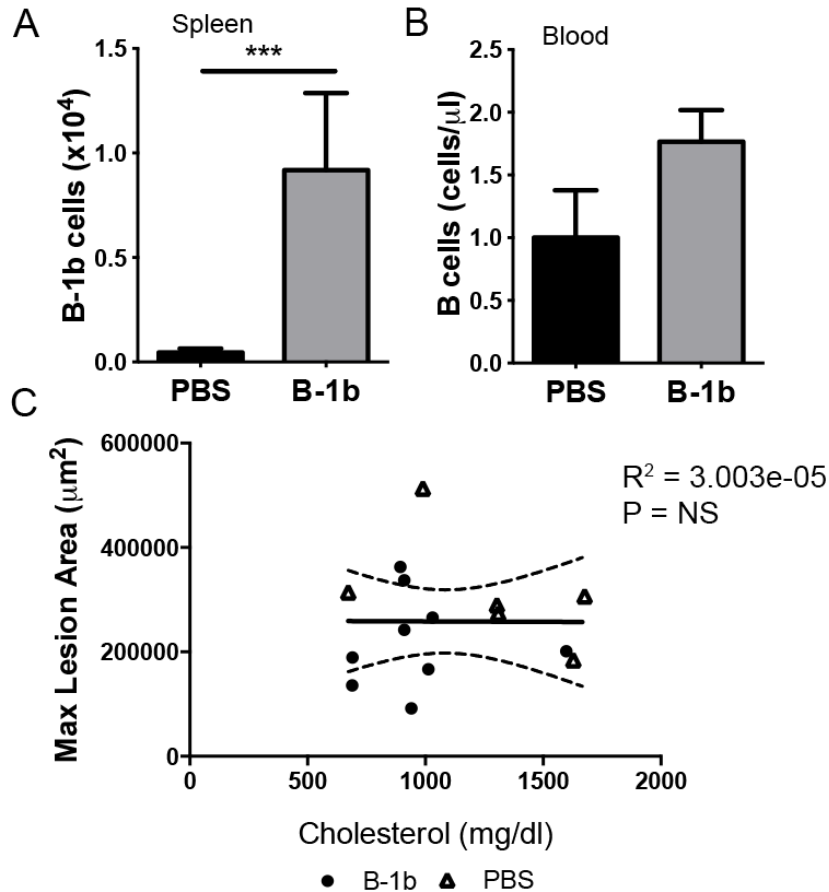
Online Figure I



Online Figure I: Standard curves and dilution series for isotype and antigen specific ELISAs. A) IgM standard curve and dilution series for total IgM, MDA-LDL IgM, and CuOx-LDL IgM used for **Figures 1B-D**. **B)** IgM standard curve and dilution series for total IgM, MDA-LDL IgM, and CuOx-LDL IgM used for **Figures 1F-H**. **C)** Standard curves for IgM, IgG1, IgG2c, and IgG3 used to calculate absolute titers for **Figure 2D** and **Figure 6A**. **D)** Dilution series for IgM, IgG1, IgG2c, IgG3, MDA-LDL IgM, CuOx-LDL IgM, native LDL, T15/E06, and 1,3,α-Dextran used for **Figures 2D-E**. **E)** Standard curves for IgM, IgG1, IgG2c, and IgG3 used to calculate

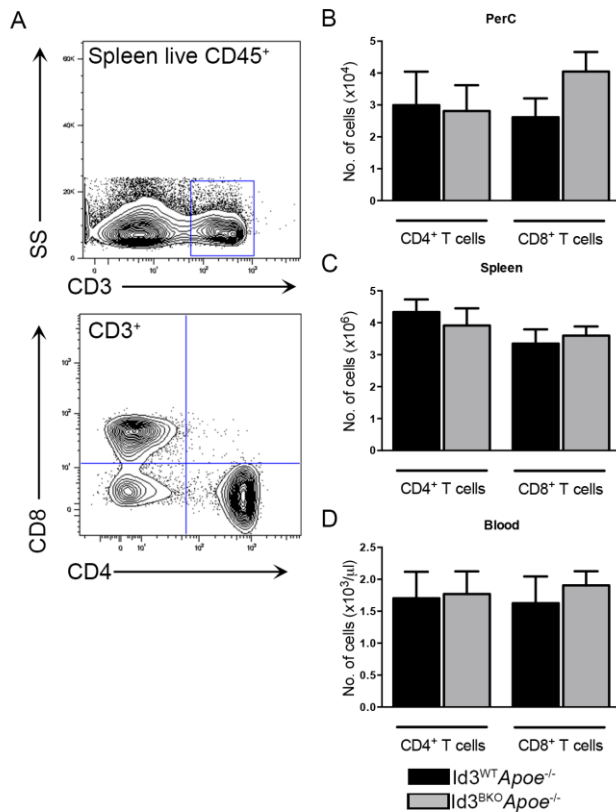
absolute titers for **Figure 2D** and **Figure 6A**. **D**) Dilution series for IgM, IgG1, IgG2c, IgG3, MDA-LDL IgM, CuOx-LDL IgM, native LDL, T15/E06, and 1,3, α -Dextran used for **Figures 2D-E** **E**) Dilution series for IgM, IgG1, IgG2c, IgG3, MDA-LDL IgM, CuOx-LDL IgM, native LDL, T15/E06, and 1,3, α -Dextran used for **Figures 6A-B**

Online Figure II



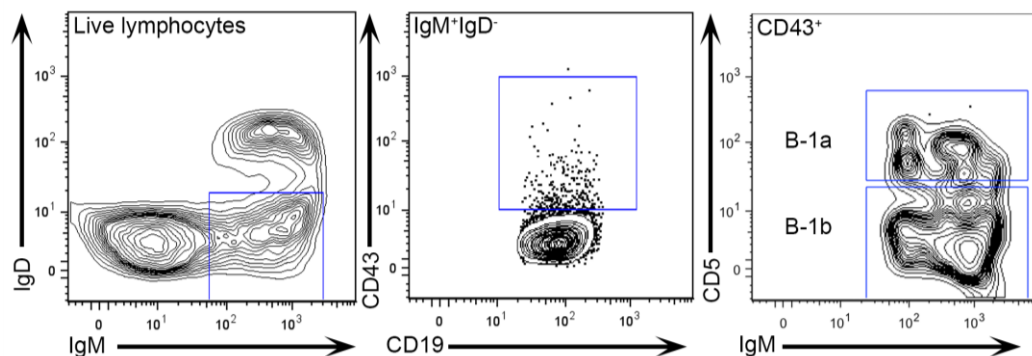
Online Figure II: B-1b cells are found in the spleen of *Rag1*^{-/-}*Apoe*^{-/-} host mice but not in blood and plasma cholesterol does not correlate with lesion area from aortic sinus. B-1b cell quantification after collection from *Rag1*^{-/-}*Apoe*^{-/-} host fed Western diet for 16 weeks from the (A) spleen and (B) blood. (C) Analysis of correlation between total plasma cholesterol (mg/dl) and max lesion area (μ m²). Data are mean \pm SEM for B-1b cell numbers and individual XY values with linear regression (solid line) and 95% confidence interval (dotted lines) for correlation analysis. Two-tailed students T-test was used to compare B-1 b cell numbers, linear regression was used for correlation analysis. ***p<0.001, NS = non-significant

Online Figure III



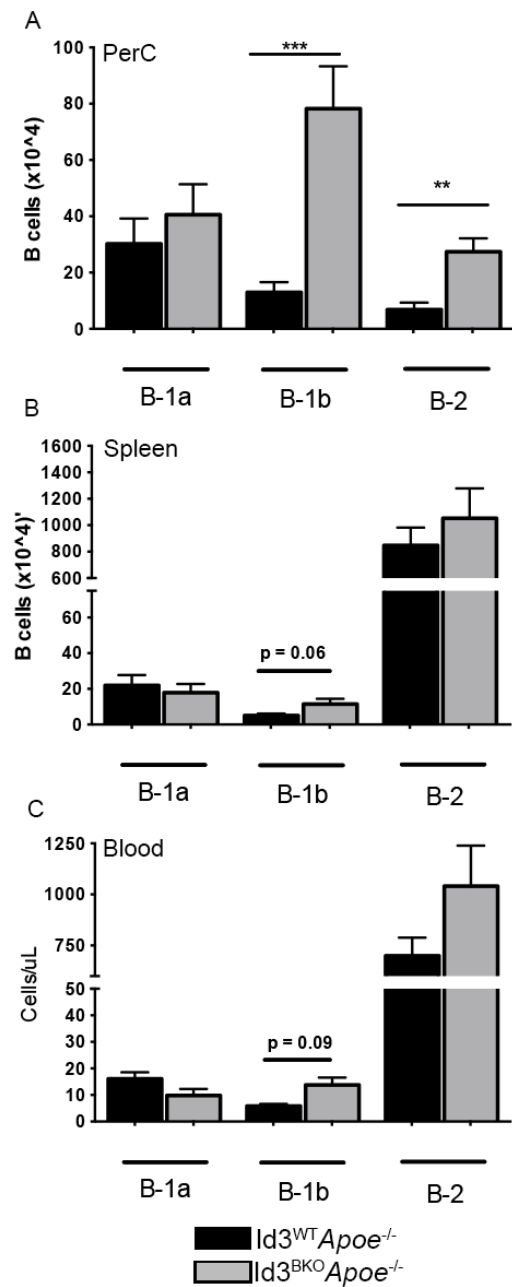
Online Figure III: Comparison of CD4⁺ and CD8⁺ T cells from 8-week-old, chow fed, male Id3^{BKO} Apoe^{-/-} and Id3^{WT} Apoe^{-/-} mice. A) Representative flow cytometry gating of splenic CD45⁺ cells then gated on CD3⁺ population and then gated on CD4⁺ or CD8⁺ populations. **B-D)** Quantification of T cell subsets from PerC (**B**), spleen (**C**), and blood (**D**). Data are mean ± SEM.

Online Figure IV



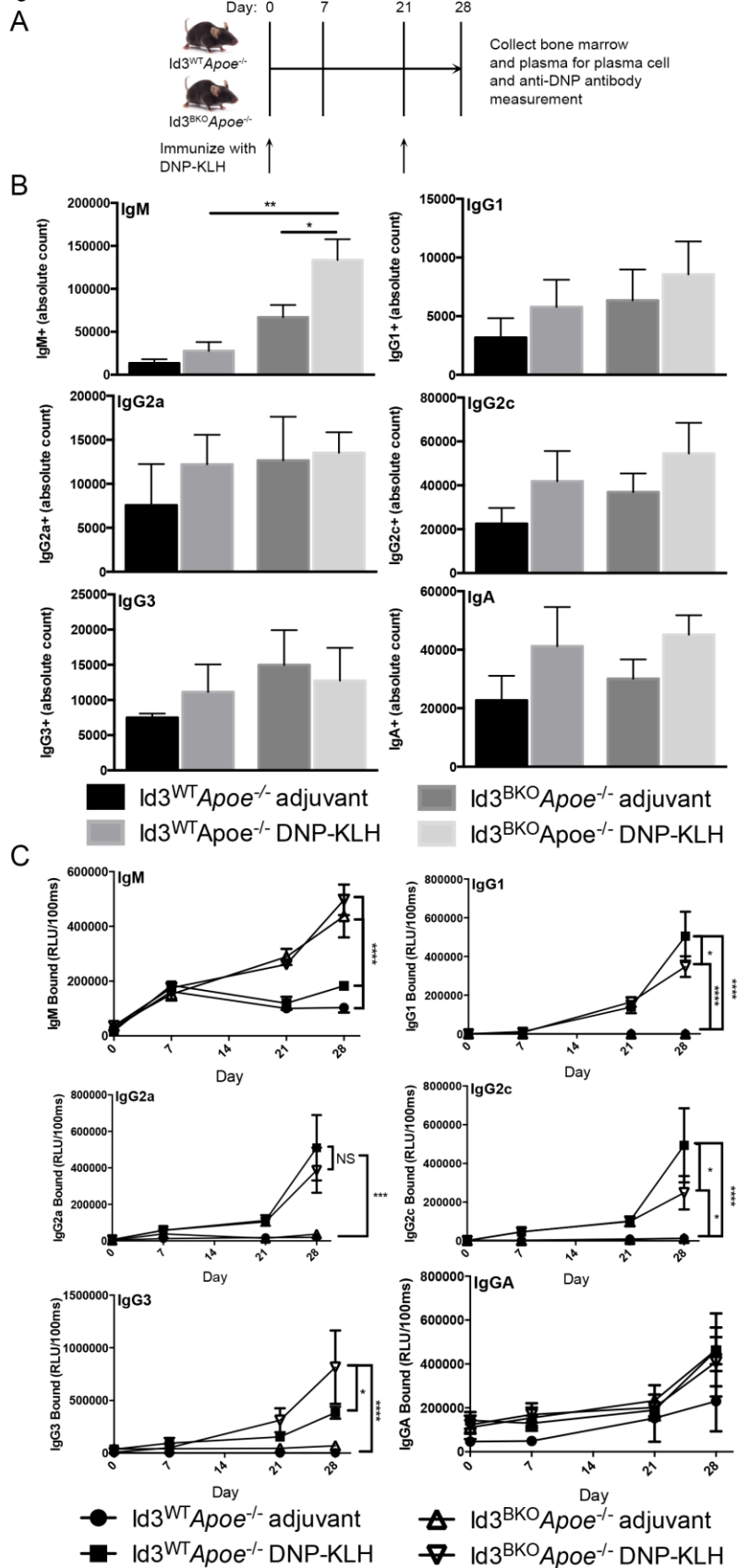
Online Figure IV: Representative gating of bone marrow B-1a and B-1b cells. Adapted from the gating described in Choi et al, *Eur J Immunol*, 2012.

Online Figure V



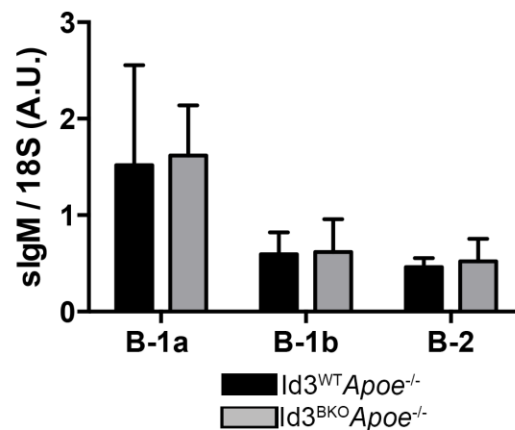
Online Figure V: Quantification of B cell subsets from PerC, spleen, and blood recovered from 16-week Western Diet fed $\text{Id3}^{\text{WT}}\text{Apoe}^{-/-}$ and $\text{Id3}^{\text{BKO}}\text{Apoe}^{-/-}$ mice. Data are mean \pm SEM. Two-tailed Students T-test was used to compare differences between genotypes. **p<0.01, ***p<0.001

Online Figure VI



Online Figure VI: $\text{Id3}^{\text{BKO}}\text{Apoe}^{-/-}$ mice respond to T cell dependent immunization with increased IgM and IgG3 and slightly blunted IgG1 and IgG2c compared to $\text{Id3}^{\text{WT}}\text{Apoe}^{-/-}$ mice. A) Study design. 8-10 week-old $\text{Id3}^{\text{BKO}}\text{Apoe}^{-/-}$ and $\text{Id3}^{\text{WT}}\text{Apoe}^{-/-}$ were immunized with 100 μg DNP-KLH in complete Freund's adjuvant ($\text{Id3}^{\text{BKO}}\text{Apoe}^{-/-}$ $n = 5$, $\text{Id3}^{\text{WT}}\text{Apoe}^{-/-}$ $n = 5$) or complete Freund's adjuvant alone ($\text{Id3}^{\text{BKO}}\text{Apoe}^{-/-}$ $n = 5$, $\text{Id3}^{\text{WT}}\text{Apoe}^{-/-}$ $n = 3$) then boosted at day 21 with DNP-KLH in PBS or PBS alone. Blood was drawn on days 0, 7, 21, and 28 (days 0 and 21 prior to immunization). Bone marrow was collected from mice on day 28 and isotype specific PCs were measured by flow cytometry. B) Absolute counts of isotype specific PCs from DNP-KLH, or adjuvant control, immunized mice. Data presented are mean \pm SEM. C) Relative anti-DNP antibody measurements determined by ELISA using isotype specific secondary antibodies for IgM, IgG1, IgG2a, IgG2c, IgG3, and IgA. Data are presented as the mean of repeat measures over time \pm SEM. One-way ANOVA and Tukey's multiple comparisons test were used to compare differences in PC counts. Two-way ANOVA and Tukey's multiple comparisons test were used to compare differences across groups on individual days. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Online Figure VII



Online Figure VII: Loss of Id3 in B cells does not modify the amount of IgM expressed per cell. Expression analysis of IgM mRNA transcripts was done in $\text{Id3}^{\text{BKO}}\text{Apoe}^{-/-}$ and $\text{Id3}^{\text{WT}}\text{Apoe}^{-/-}$ PerC B cell subsets and normalized to the housekeeping gene 18S rRNA. Data are mean \pm SEM.

Online Table I: Weight and lipids from *Rag*^{-/-} *Apoe*^{-/-} mice injected with B-1b cells (or PBS control) and fed a Western diet for 16 weeks

Injection	PBS (n=6)	B-1b cells (n= 9)	P
Weight (g)	36.09± 1.87	32.89± 1.90	NS
Weight gain (g)	11.55± 0.66	8.05± 2.34	NS
Total Cholesterol (mg/dL)	1247± 162	964± 89	NS
Triglycerides (mg/dL)	262.3± 29.43	223.3± 29.34	NS
HDL (mg/dL)	46.50± 2.63	36.67± 4.63	NS

Online Table II: Weight and lipids from *Id3*^{WT} and *Id3*^{BKO} mice fed a Western diet for 16 weeks

Genotype	<i>Id3</i>^{WT} (n= 11)	<i>Id3</i>^{BKO} (n= 7)	P
Weight (g)	38.62± 0.89	40.08± 0.86	NS
Weight gain (g)	12.52± 1.35	14.56± 0.93	NS
Total Cholesterol (mg/dL)	1476± 65.0	1514± 100.3	NS
Triglycerides (mg/dL)	358.6± 23.3	387.9± 29.2	NS
HDL (mg/dL)	43.64± 3.19	42.60± 4.13	NS

Works Cited

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